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**EFFECT OF DUCT OBSTRUCTION ON STRUCTURE, ELEMENTAL COMPOSITION,
AND FUNCTION OF RAT SUBMANDIBULAR GLANDS**

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Abstract

Obstruction of salivary glands occurs in association with a number of pathological conditions. It has been suggested that the major changes found in the salivary glands of patients with cystic fibrosis are due to obstruction of the excretory duct by viscous mucus. In the present study, the effect of excretory duct obstruction on structure, elemental composition and function of rat submandibular gland was investigated. Obstruction was effected by infusion of a fast-hardening protein emulsion in the main excretory duct. After 1 week, and more pronounced after 2 weeks of obstruction the number of granular duct cells had decreased in the obstructed gland. X-ray microanalysis showed an increase in Mg, Ca and K, and a decrease in Na levels in the acinar cells, compared to normal glands. The contralateral glands apparently underwent compensatory hypertrophy and showed a similar pattern of changes in elemental composition. The composition of pilocarpine-induced submandibular saliva was neither in the obstructed nor in the contralateral gland significantly different from that in control glands. However, the flow rate was somewhat lower. Hence, increase in cellular Ca levels in submandibular gland acinar cells in cystic fibrosis could be secondary to duct obstruction, but the present study does not support the hypothesis that duct obstruction would result in changes in the composition of saliva.

Key Words: salivary glands, obstruction, secretion, mucus, cystic fibrosis.

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Introduction

Obstruction of salivary glands occurs in association with a number of pathological conditions, and obstructive adenitis following salivary stones or infectious disease of the gland is not a rare disease. Several histological and ultrastructural investigations on the effect of obstruction of salivary ducts in experimental animals have been carried out; the rat submandibular gland has been studied by e.g., Shiba et al. (1972), Tamarin (1979), and Norberg et al. (1988).

Cystic fibrosis is a hereditary, inborn generalized exocrinopathy with chronic obstructive lung disease and pancreatic insufficiency as primary clinical symptoms. The severe structural and functional changes in the pancreas are assumed to be due to degeneration of the exocrine tissue after obstruction of the duct by viscous secretions. In cystic fibrosis, also the salivary glands appear to be affected, even though the changes in this tissue are of little clinical significance. According to Tandler (1987), most of the changes in the salivary glands should be regarded as secondary to obstruction.

In the present study, the effect of obstruction of the submandibular gland duct on the histology, ultrastructure, elemental composition and function of the gland was studied. In contrast to previous studies, the obstruction was not effected by ligation, but by infusion with a fast-hardening protein solution resulting in the formation of a protein clot obstructing the distal part of the duct.

Materials and Methods

A total of 24 male Sprague-Dawley rats weighing 175-200 g were used in the obstruction experiments. The animals were lightly anesthetized with sodium barbital and with a fine syringe a drop of Ethibloc^R (Ethicon GmbH, Norderstedt, FRG) emulsion

was brought into the right submandibular duct. Ethibloc is a fast-hardening protein emulsion used for the occlusion of pancreatic ducts and sealing off of blood vessels in surgery. The animals were then kept under standard conditions with access to food and water *ad libitum*, for a period of 1 or 2 weeks, as specified under Results.

From some animals, the obstructed and the contralateral gland were removed for histological, ultrastructural and X-ray microanalytical studies. These animals were deprived of food the night before sacrifice. The submandibular glands were removed under heavy pentobarbital anesthesia. For light (LM) and transmission electron microscopical (TEM) investigations, small pieces of the gland were fixed in cacodylate-buffered glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Polarbed 812 resin (Polaron, Watford, U.K.). Semi-thick sections for light microscopy were stained with toluidine blue. Thin sections for TEM were contrasted with uranyl acetate and lead citrate, and studied at 60 kV in a JEOL 100S electron microscope.

For X-ray microanalysis, small pieces of the gland were rapidly frozen in liquid nitrogen. Thick (16 μ m) cryosections were cut at -30°C on a conventional cryostat, mounted on polished graphite planchets, freeze-dried overnight in the cryostat, and coated with a conductive carbon layer (Wróblewski et al., 1987). The specimens were analyzed with a Tracor 5500 energy-dispersive X-ray detector system mounted on a JEOL 1200EX electron microscope. The specimens were viewed in the secondary mode and analyzed at an accelerating voltage of 20 kV. Elemental concentrations were calculated by measuring the ratio (P/B ratio) of the characteristic peak to the continuum in the same energy range (the background under the peak) in the specimens and in a standard. The standard consisted of mineral salts in known concentration dissolved in a 20% gelatin/5% glycerol matrix, frozen, sectioned and analyzed in the same way as the specimen (Müller and Roomans, 1985; Roomans, 1988).

The remaining animals were used for physiological studies. These animals were not deprived of food overnight. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), and tracheotomized to provide clear airways during cannulation. The animals were placed on a heating pad to maintain a body temperature of 37°C. A fine polyethylene cannula (Clay Adams PE10) drawn to a tip diameter of about 100 micrometers was inserted extraorally in the main excretory ducts (proximal to the clotted Ethibloc in the obstructed ducts) after dissection in a dissection microscope. Salivation was induced by pilocarpine

(8 mg/kg body weight). The saliva produced in the initial 4 min was discarded, the saliva produced in the subsequent 10 min was collected, and the amount of saliva determined gravimetrically. After the end of the collection period, the glands were removed, separated from the sublingual gland, and weighed. The saliva samples were analyzed for Na, K, and Ca by means of atomic absorption spectrometry and for protein by the method of Lowry et al. (1951).

The data obtained from the obstructed glands and the contralateral glands were compared to data obtained on glands from animals that had not been treated in any way, but had been kept under comparable conditions in the laboratory.

Results

Light microscopical investigations showed that in the obstructed glands, the secretory granules of the granular duct cells were absent (Fig 1). Transmission electron microscopy showed that in comparison to the control gland (untreated animals) the acinar cells of the obstructed and contralateral gland contain somewhat increased amounts of intracellular mucus (Fig 2). Morphometrical studies showed that the relative volume of mucus in the acinar cells, which was 40% in the control animals, increased to 46 % in the obstructed gland and 47 % in the contralateral gland. Typical X-ray spectra of submandibular gland acinar cells are shown in Fig 3. Quantitative microanalysis of mucous acinar cells showed a decrease in Na, and an increase in Mg, K and Ca concentration in both the obstructed and the contralateral gland and compared to the untreated control. The changes in the contralateral gland were more pronounced than those in the obstructed gland (Fig 4).

The flow rate of pilocarpine-induced saliva was similar for the obstructed and the contralateral glands, but somewhat lower than in the control glands. The data on flow rate, and on Na, K, Ca, and protein concentration in the submandibular saliva are summarized in Table 1.

Discussion

Shiba et al. (1972) and Norberg et al. (1988) observed acinar dystrophy after ligation of the submandibular duct. A decreased size of the mucous acini in obstructed glands would thus be expected. In our study, however, the effect appears less dramatic than in previous investigations. This may be due to the difference in technique of obstruction; it cannot be excluded that the Ethibloc obstruction is less absolute than ligation of the duct.

Effects of Salivary Duct Obstruction

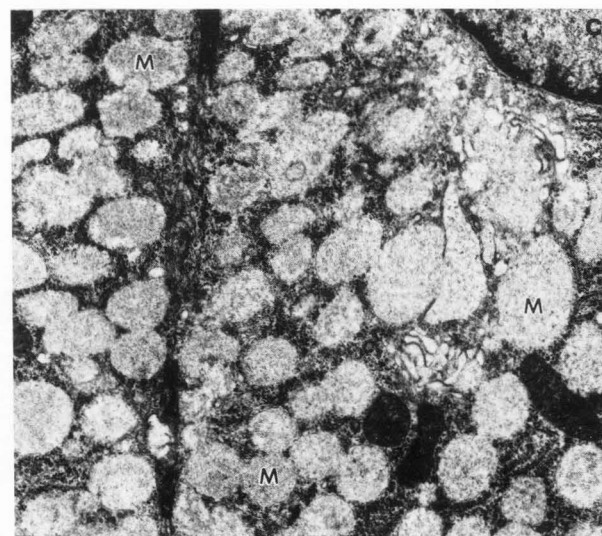
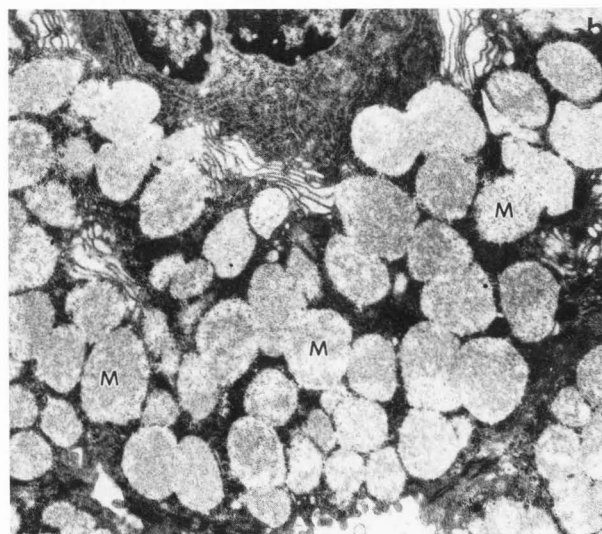
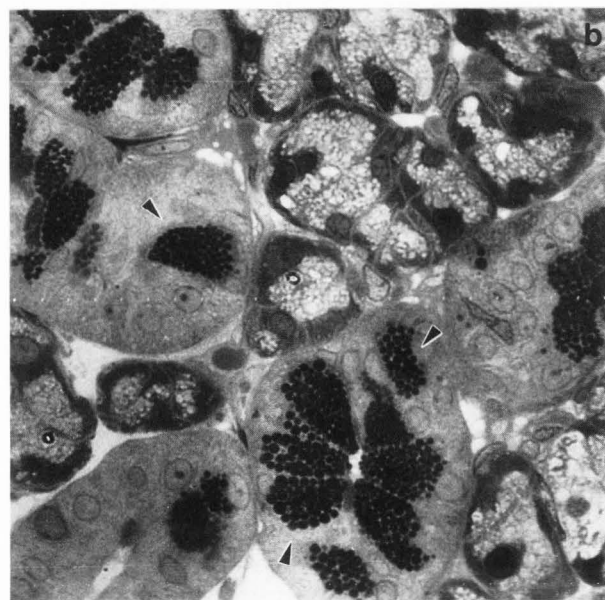
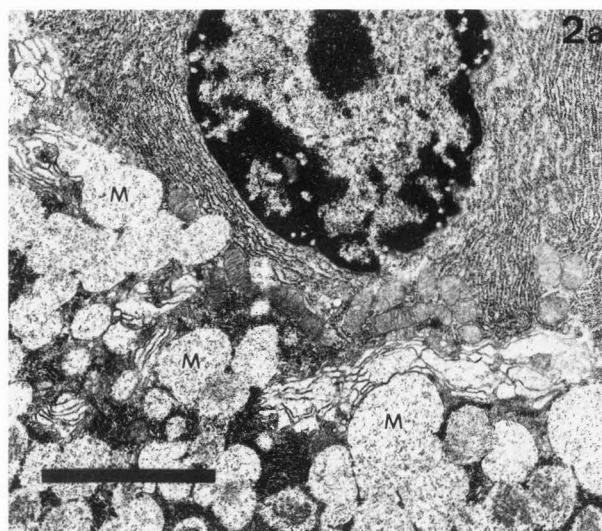
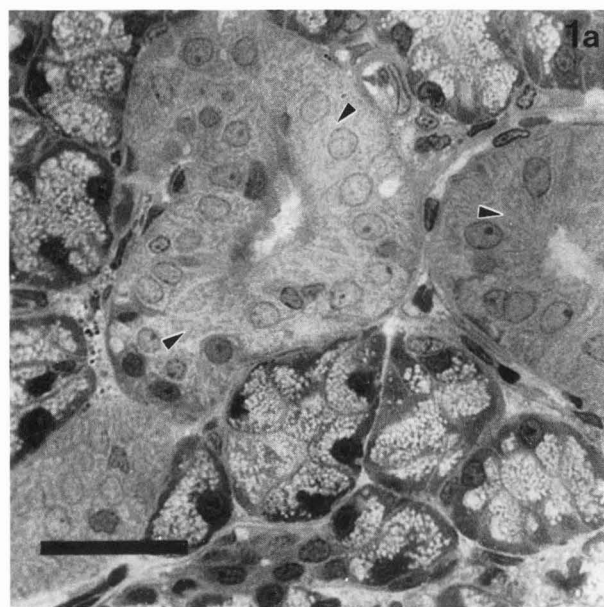


Fig. 1. Light micrographs of (a) obstructed gland, (b) contralateral gland, (2 weeks of obstruction) showing lack of serous granula in the granular duct cells of the obstructed gland (arrows). Bar = 50 μ m.

Fig. 2. Transmission electron micrograph of a mucous acinar cell (a) of a control gland, (b) of the contralateral gland (2 weeks of obstruction), (c) of the obstructed gland (2 weeks of obstruction). In (b) and (c) the amount of mucus is increased. M = mucus granule. Bar = 5 μ m.

Table 1

Composition and flow-rate of pilocarpine-induced submandibular saliva in rat

	<u>control</u>	<u>obstructed</u>	<u>contralateral</u>
flow rate (ml/min/g)	79 ± 4	62 ± 6*	64 ± 5*
Ca (mM)	0.9 ± 0.04	0.78 ± 0.12	0.85 ± 0.11
Na (mM)	2.1 ± 0.4	2.2 ± 0.5	2.5 ± 0.8
K (mM)	70 ± 4	62 ± 4	64 ± 5
protein (mg/ml)	4.1 ± 0.3	3.3 ± 0.5	3.8 ± 0.5

The data represent mean and standard error of 23 control glands, 6 obstructed glands and 8 contralateral glands. Statistical significance of the difference between control and obstructed or contralateral glands ($p < 0.05$) is indicated by an asterisk (*).

Nonetheless, we consider our method preferable because it greatly reduces the experimental trauma suffered by the animals and in addition, it may more closely reflect the situation in obstruction of the human gland both in cystic fibrosis and in other diseases involving salivary duct obstruction. Our findings also confirm the observation of Norberg et al. (1988) of degranulation of granular duct cells after obstruction, which in itself is proof of the effectiveness of the Ethibloc obstruction. Since the granular duct cells are typical for rats and some other rodents, and do not occur in man, it is difficult to assign any significance to this phenomenon in human oral pathology.

Significant changes were found in the elemental composition of the mucous acinar cells in both obstructed and contralateral glands. In both cases, Mg and Ca were increased and Na decreased. These changes reflect an increased relative mucus content of the acinar cells: the intracellular mucus is relatively rich in Ca, and to a lesser extent in Mg. A relative increase of the amount of intracellular mucus therefore results in an increase in the cellular Mg and Ca concentration. The same pattern of elemental changes is observed in the chronically isoproterenol-treated rat (an animal model for cystic fibrosis) (Müller and Roomans, 1985) and, indeed, in a number of experimental animal models with relevance for cystic fibrosis (Roomans, 1986; Roomans et al., 1989). Increased calcium levels have also been observed in submandibular gland acinar cells of patients with cystic fibrosis (Roomans et al., 1989). Although the similarity between the effect of obstruction and the situation in the cystic fibrosis gland is no absolute proof that the increase in intracellular Ca level is due to obstruction of the excretory duct system, our findings provide an indication

that the changes in Ca levels are not necessarily close to the primary defect in cystic fibrosis.

It is striking that the elemental changes are actually more pronounced in the contralateral gland than in the obstructed gland. This has not been appreciated in earlier studies. Given the time period of 1 or 2 weeks between the obstruction and the sacrifice of the animals, it is unlikely that the brief and light anesthesia necessary to introduce the protein plug in the duct is the reason for the difference between the contralateral gland and the glands in untreated control animals. Rather, a compensatory hypertrophy of the contralateral gland appears indicated.

There is some difference of opinion in the literature about the functional condition of the obstructed gland. Our data indicate a near-normal flow rate after pilocarpine stimulation, which is in agreement with findings of Junqueira and Rabinovitch (1954) and Bhaskar et al. (1966) that the obstructed gland can recover rapidly after recanalization of the duct.

Diverging opinions are also found with regard to changes in the elemental composition of the submandibular saliva of patients with cystic fibrosis (reviewed by Quinton, 1984). However, most studies appear to show increased levels of Na, Ca and protein in cystic fibrosis submandibular saliva (e.g., Ceder et al., 1983). In the present study significant changes in ion or protein levels could not be detected, neither in saliva elicited from the obstructed, nor from the contralateral gland. The changes in submandibular saliva composition in cystic fibrosis patients can thus not be explained by obstruction or occlusion of the excretory duct system.

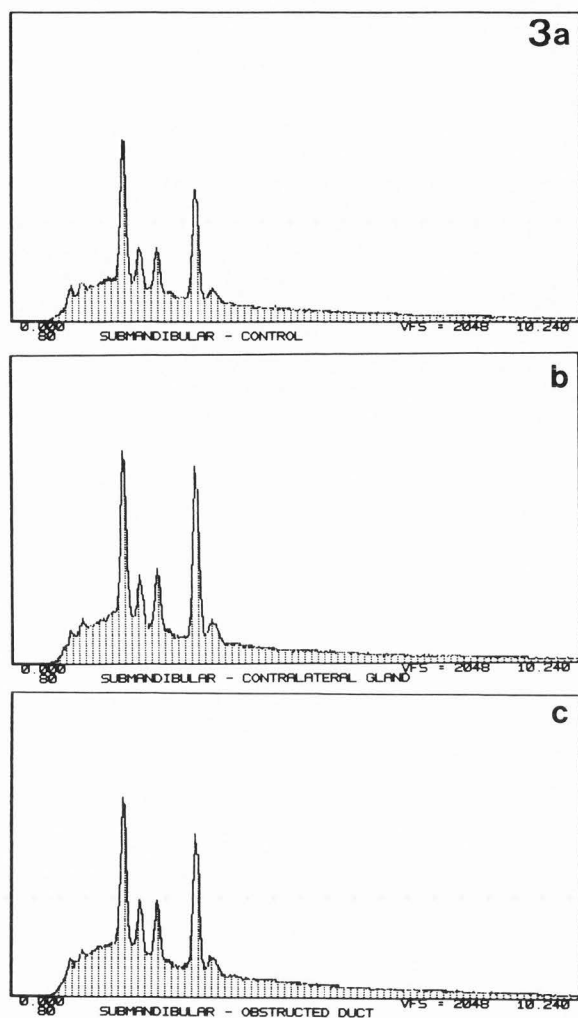


Fig. 3. Typical energy-dispersive X-ray spectra of submandibular gland acinar cells (a) of a control gland, (b) of a contralateral and (c) of an obstructed gland.

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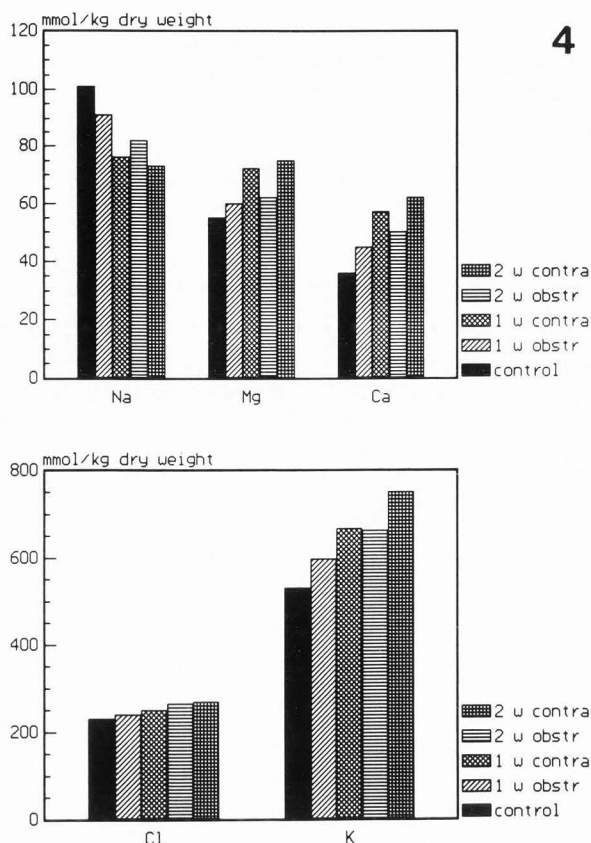


Fig. 4. Elemental concentrations in mucous acinar cells of the submandibular gland in control (untreated) animals, and in obstructed and contralateral glands (1 and 2 weeks).

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Discussion with Reviewers

S.H. Ashrafi: How much protein (Ethibloc) was used to obstruct the submandibular duct?

Authors: The exact amount was not determined but we estimate that about 0.04 ml of protein solution was used.

S.H. Ashrafi: What was the advantage of freezing tissue directly in liquid nitrogen without using isopentane (intermediate immersion medium)? In our experiments we have seen more ice-crystal formation when tissue is directly frozen in liquid nitrogen.

Authors: Freezing directly in liquid nitrogen is simpler. Since with 16 μ m thick cryostat sections the level of resolution is at the cellular level, the size of the ice crystals is not critical. However, we agree that freezing in other cryogens cooled by liquid nitrogen, rather than in liquid nitrogen itself, may result in smaller ice crystals.

S.H. Ashrafi: Did you use any adhesive to stick the sections on graphite planchets?

Authors: No. The sections were made to adhere to the planchet by very slightly warming the planchet with a finger under the section as described in Wróblewski et al. (1987).

S.H. Ashrafi: Could you explain the reasons beside hypertrophy why elemental changes were more pronounced in the con-

tralateral gland than in the obstructed gland?

G.A.J. Kuijpers: It is striking that acinar dystrophy of the obstructed glands and compensatory hypertrophy of the contralateral gland (as suggested in the Discussion) would be paralleled by similar structural and elemental changes. Could you comment on this?

Authors: Regardless of the changes in size of cells and acini, the increase in relative mucus content of the cells is the factor determining the increase in cellular calcium levels. Accumulation of intracellular mucus appears to be an unspecific consequence of any disturbance in gland function (Roomans et al., 1989). In past experiments, we have, e.g., shown that both acidosis and alkalosis produce the same effects on gland structure and elemental composition (Roomans, 1986). The hypertrophy of the contralateral gland appears to us sufficient explanation for the differences observed between the contralateral and the control gland. The differences between the contralateral gland and the obstructed gland are of a quantitative nature.

G.A.J. Kuijpers: How do you know that the elements you measure are in the acinar and not in the (abundant) ductular cells of the gland?

Authors: The mucous acinar cells can easily be distinguished from the duct cells by their X-ray spectrum. Duct cells typically have much higher S and lower Ca values than acinar cells (Roomans et al., 1989). In addition, the ducts can be seen in the scanning microscope image of the cryosection.

G.A.J. Kuijpers: Does the decreased Na concentration also reflect an increased mucus content of the acinar cells? And how do you explain the increase in K concentration? Since you are measuring elemental concentrations in thick cryosections, could it be that a decrease of the relative volume of extracellular space leads to the decrease of the Na signal and increase of the K signal in the treated animal?

Authors: We also measured an increase in P concentration in the acinar cells of the contralateral (955 mmol/kg dry weight) and obstructed gland (816 mmol/kg dry weight) as compared to the control glands (715 mmol/kg dry weight). This indicates an increase in P-containing compounds in the acinar cell, presumably nucleotides. Since we have not carried out analysis at the subcellular level we do not know to which cellular compartment this increase is due. Nucleotides preferentially bind K over Na and therefore the increased P could explain the increased K and decreased Na. The volume of analysis should be comprised within one cell and changes in the relative volume of extracellular space should not affect the results.